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# **Title: Immune response to Mycobacterium tuberculosis in young contacts with discordant immunological test results**

## **Running title: Biomarkers in childhood tuberculosis**

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Sir,

Concerns regarding the use of quantiFERON (QFT) in children younger than 5 years old include a higher rate of indeterminate results and the potentially lower sensitivity of QFT as a result of an impaired capacity to secrete IFN- $\gamma$  in young age [1].

In this Journal, we recently reported that impaired cytokine responsiveness that resembles the concept of immunoparalysis was responsible for indeterminate QFT results in young children with clinical signs compatible with tuberculosis (TB) but with the final diagnosis of infectious diseases different from TB [2]. In our area (Ile de France, France) where BCG vaccination is recommended soon after birth we observed a significant discordance between Tuberculin-Skin Test (TST) positivity and QFT negativity in young children recently exposed to household TB cases. Based on the hypothesis of imperfect QFT sensitivity, here cytokine/chemokine responses to *Mycobacterium tuberculosis* (M.tb) were evaluated in immunocompetent 0-5 years old TST<sup>+</sup>/QFT<sup>-</sup> contacts with the goal of improving QFT sensitivity.

Among 105 consecutive 0-5 years old immunocompetent children who were referred to our center as recent household contacts from June 2012 to September 2014, ten had TB disease diagnosis based on chest x-ray abnormalities compatible with TB and favorable outcome on anti-TB treatment. The correlation between QFT and TST results was excellent in this group (QFT<sup>+</sup>/TST<sup>+</sup>=9/10,  $k=1$ , 95% CI [1 to 1]). QFT and TST results showed however a very poor correlation among 26 asymptomatic children with latent-TB diagnosis based on TST and/or QFT positivity while chest x-ray was normal ( $n=19/26$  discordant results,  $k=-0.16$ , 95% CI [-0.378 to +0.059]). Among discordant results, the TST<sup>+</sup>/QFT<sup>-</sup> profile was the most common (17/19). Cytokine/chemokine responses to M.tb were measured in 15 of these 17 children (median age: 2y 5m, BCG vaccinated 15/15). In addition, two populations of reference were selected among the same series to identify biomarkers of infection and to

determine their cut-offs of positivity. The first population named true-positives (n=8, BCG-vaccinated n=7/8) were age-matched (median age: 2y 3m), TST<sup>+</sup>/QFT<sup>+</sup> infected children (uncomplicated thoracic TB: n=6/8, latent-TB: n=2/8). The second population named true-negatives (n=8, BCG-vaccinated n=6/8) were age-matched (2y 9m) uninfected children with no clinical sign of TB, normal chest x-ray and negative results for both TST and QFT following an exposure to a not infectious (non-bacillary) index case. Eighteen cytokines/chemokines were measured in the residual plasma of the three QFT (quantiferon-GIT, Cellestis) tubes i.e. unstimulated, PHA-stimulated and M.tb-stimulated tubes from any subject as previously described [2, 3].

Five biomarkers showing either concentrations lying outside an interpretable range (IL-8, MCP-1 and MIP-1 $\beta$ ) or similar concentrations (IL-7 and IL-12) in the 3 QFT tubes were excluded from analysis. In true-positives, three cytokines (IL-2, IL-5 and IL-13) and one chemokine (IP-10) were significantly more elevated in the M.tb peptide-stimulated opposed to the unstimulated (background) tubes (table 1). Receiver operating characteristics (ROC) curves were generated by plotting biomarker release values observed in true negatives (specificity) against the corresponding values in true positives (sensitivity). In agreement with the current literature, individual concentrations detected in the tubes containing M.tb peptides minus background were used for this analysis. As illustrated in figure 1A, the only IL-2, IL-5, IL-13 and IP-10, in addition to IFN- $\gamma$  (p=0.0007), displayed a significant ability to discriminate between true-positives and true-negatives.

In TST<sup>+</sup>/QFT<sup>-</sup> contacts, the IL-13 and to a lesser extent the IP-10 displayed a higher level of expression in the M.tb peptide-stimulated than in the background QFT tubes (Table 1). As illustrated in figure 1B, ROC analysis confirmed that the only IL-13 and IP-10 could discriminate TST<sup>+</sup>/QFT<sup>-</sup> contacts from true-negatives. Note in this figure that the threshold

cut-off values of IL-13 and IP-10 that best discriminated true-positives and QFT<sup>+</sup>/TST<sup>-</sup> contacts from true-negatives (Younden's index) were highly similar.

The threshold values that best discriminated true-positives from true-negatives were used to categorize TST<sup>+</sup>/QFT<sup>-</sup> contacts as responders or non-responders. IP-10 identified eight (53%) and IL-13 identified ten (67%), TST<sup>+</sup>/QFT<sup>-</sup> contacts as responders. Combining IL-13 and IP-10 increased the number of TST<sup>+</sup>/QFT<sup>-</sup> contacts classified as responders to 12/15 (80%). IL-2 and IL-5 did not identify additional responders among IL-13 and/or IP-10 positive TST<sup>+</sup>/QFT<sup>-</sup> contacts.

This study provides important information regarding M.tb immunity in young children and may provide a preliminary basis for further improvement of immunodiagnostic tests for pediatric latent-TB.

Higher susceptibility to TB in young children has been attributed to the propensity for infants to develop poor IFN- $\gamma$  responses but relatively preserved Th2 responses to immunogens [4]. Solid evidence to support this hypothesis in M.tb infection is lacking. Low M.tb-specific IFN- $\gamma$  response associated with the abundant M.tb-specific IL-13 response in 0-5 year-old TST<sup>+</sup>/QFT<sup>-</sup> children with latent-TB (contained infection) does not support this dogma and fit with the most recent concept that components of a successful immune response to M.tb involve more than just IFN- $\gamma$  and are largely unknown [5].

As far as we know, this study is the first that has analyzed multiple cytokines with the goal of identifying alternative/additional cytokines that define M.tb infection selectively in 0 - 5 year old children. Our results, showing that 8/15 TST<sup>+</sup>/QFT<sup>-</sup> children screened positive with our IP-10 release assay, agree with the concept that combining IFN- $\gamma$  and IP-10 testing may be one approach to increase the sensitivity of QFT in young children [6]. Our results also agree with previous studies showing an IL-13 response to M.tb peptides in pediatric TB [7-9]. Yet, this study first reports the potential added value of IL-13 in diagnosing M.tb infection in

young children. Overall, only 9/26 children with the diagnosis of latent-TB from our series displayed QFT positivity (sensitivity 35%). Adding the ten children with QFT negativity but IL-13 positivity improved sensitivity to 73%. Combining IL-13 and IP-10 further improved sensitivity to 81%. These results engage to further compare head to head the accuracy of IP-10 and also of IL-13 as biomarkers with IFN- $\gamma$  in large samples of healthy pediatric contacts. At best, these future studies should focus on the performance of these biomarkers according to age, TB status and BCG status to define the pediatric subgroups that could best benefit from this new approach.

## **Conflict of interest**

There is no financial support, grants, financial interests or consultancy that could lead to a conflict of interest. All authors state that they have read and approved the manuscript. The results have not been published elsewhere nor are they under consideration for publication elsewhere nor they have been presented at any conference.

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**Table 1: Biomarker concentrations (pg/ml) in Background and M.tb peptide-stimulated whole bloods**

Biomarkers	True-negatives (n=8)			True-positives (n=8)			TST <sup>+</sup> /QFT <sup>+</sup> contacts (n=15)		
	Background	M.tb peptides	p <sup>(*)</sup>	Background	M.tb peptides	p <sup>(*)</sup>	Background	M.tb peptides	p <sup>(*)</sup>
<b>IL-2</b>	206 (137-1193)	250 (116-706)	0.7241	110 (45-233)	3350 (576-7152)	<b>0.0001</b>	160 (74-415)	180 (98-829)	0.3445
<b>IL-4</b>	146 (123-218)	152 (97-237)	1	76 (50-181)	140 (87-321)	0.0823	130 (75-456)	137 (87-454)	0.3921
<b>IL-5</b>	22 (11-37)	24 (17-67)	0.6415	15 (8-46)	81 (33-4822)	<b>0.0021</b>	18 (13-67)	20 (15-70)	0.1225
<b>IL-6</b>	8827 (3164-14243)	8492 (1662-12523)	0.7289	3548 (1552-20359)	5849 (3300-24632)	0.2754	4904 (1184-14243)	6066 (1867-11235)	0.4863
<b>IL-10</b>	186 (126-526)	163 (103-221)	0.1941	76 (30-376)	80 (32-422)	0.7981	128 (80-526)	148 (97-337)	0.3212
<b>IL-13</b>	47 (27-434)	39 (26-429)	0.5033	37 (24-58)	579 (172-5876)	<b>0.0001</b>	24 (18-62)	131 (23-247)	<b>0.0005</b>
<b>IL-17</b>	430 (320-534)	388 (320-462)	0.3269	230 (165-402)	333 (227-567)	0.0623	374 (265-471)	385 (243-584)	0.4179
<b>IFN-g</b>	134 (91-177)	116 (73-240)	0.5258	58 (28-154)	1165 (115-1872)	<b>0.0006</b>	91 (44-584)	93 (68-576)	0.3724
<b>TNF-a</b>	9039 (1407-21766)	8113 (1522-19491)	0.7241	7224 (1562-10555)	10109 (2370-20487)	0.0684	15220 (540-21766)	14770 (348-22415)	1
<b>IP-10</b>	705 (425-1454)	1202 (484-1454)	0.4839	906 (125-2785)	17262 (700-22657)	<b>0.0016</b>	555 (126-1929)	3691 (506-11870)	0.0050
<b>G-CSF</b>	231 (78-329)	162 (77-475)	0.3241	60 (24-435)	76 (32-548)	0.6479	102 (56-329)	115 (80-271)	0.2649
<b>GM-CSF</b>	327 (175-679)	334 (188-526)	0.7986	179 (117-442)	338 (255-603)	0.0210	260 (138-702)	286 (177-591)	0.3505
<b>IL-1b</b>	5354 (2625-8934)	7119 (3591-19685)	0.3251	2749 (761-7380)	5645 (1560-21344)	0.3814	3310 (353-9141)	4580 (2218-12873)	0.1369

M.tb: *Mycobacterium tuberculosis* .PHA : phytohemagglutinin.

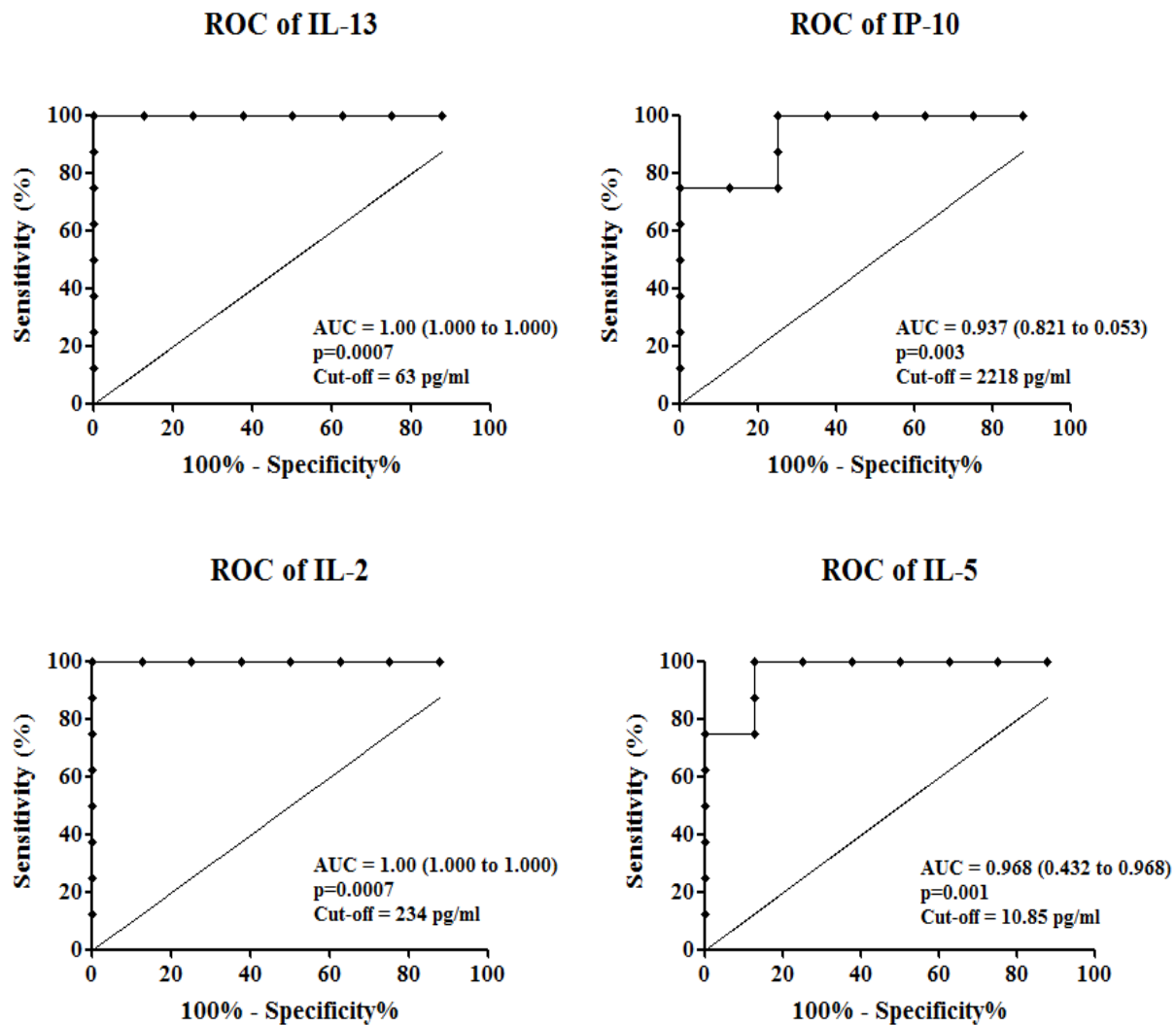
Results are given as median, (range) concentrations in pg/ml.

p-values below 0.004 thresholds (corresponding to Bonferroni adjustment for 13 tests) are highlighted in bold.

(\*) Mann-Whitney test comparing the background value with the corresponding value observed in M.tb peptide-stimulated whole bloods.

**Figure 1**

**A: True-negatives vs true-positives**



**B: True-negatives vs TST<sup>+</sup>/QTF<sup>-</sup> contacts**

